EXHIBIT B

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Assembly of functional antibodies from immunoglobulin heavy and light chains synthesised in *E. coli*

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ABSTRACT

Genes for a murine μ heavy chain and a λ light chain immunoglobulin have been inserted into bacterial expression plasmids containing the Escherichia coli trp promoter and ribosome binding site. Induction of transcription from the trp promoter results in accumulation of both light and heavy chain polypeptides in appropriate host strains. Both proteins were found as insoluble products. Following extraction and purification of the immunoglobulin containing fractions, antigen binding activity was recovered. The activity demonstrates essentially the same properties as the antibody from the hybridoma from which the genes were cloned.

INTRODUCTION

Immunoglobulin genes and their products represent one of the most extensively studied families of eukaryotic macromolecules. Immunoglobulin polypeptides are secreted proteins and are synthesised with an amino-terminal signal peptide which is cleaved to yield the mature protein. The expression of immunoglobulin genes in E. coli forms the initial stage in the production of antibodies produced via recombinant DNA techniques. Such antibodies would have many uses. For example, detailed studies on antigen-antibody interactions following alterations of the antigen combining site by site directed mutagenesis could be carried out, or the Fc regions of the molecules could be altered for specific uses such as binding to matrices for immunopurification. Thus, it is surprising that with the many studies on expression of eukaryotic genes in E. coli (1), little has been done on immunoglobulin genes. So far immunoglobulin genes have been expressed in modified forms at low levels in E. coli, usually as incomplete amino-terminal fusion proteins (2,3,4). In one case, a trpE-IgE fusion has been expressed at 10% total E. coli protein (5).

Here we describe the bacterial expression of a murine μ heavy chain and a murine λ light chain immunoglobulin cDNA. The Ig μ and λ genes used in these studies are from cDNA clones isolated from the hybridomas B1-8 and S43

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(6), respectively. These hybridomas were raised to the hapten 4-hydroxy-3-nitrophenyl acetyl (NP) and produce IgM antibodies which are termed heteroclitic, that is binding a related hapten e.g. 4-hydroxy-5-iodo-3 nitrophenyl acetyl (NIP) more strongly than NP (7). The μ gene was cloned from the hybridoma line B1-8 (8) and the λ gene was cloned from the related hybridoma S43 (9). However, the sequence of the λ from S43 varied by only two amino acids from B1-8 λ sequence, assuming that B1-8 λ has germ-line sequence (9). Both changes were conservative and outside of the complementarity determining regions. So in effect, the antibody from B1-8 can be used to represent the parental monoclonal antibody.

The two polypeptides were synthesised in E. coli as native proteins lacking eukaryotic signal sequences and presumably possessing amino-terminal methionine residues. High levels of expression were achieved using the E. coli K12 strain E103S or E. coli B but only low levels of expression occurred in HB101 (10). Following solubilisation of μ and λ polypeptides expressed in the same cell or different cells, the protein products were purified and antigen binding activity recovered. This activity demonstrates essentially the same properties as those found for an NP binding IgM hybridoma antibody.

MATERIALS AND METHODS

Chemicals and Cloning Procedures

Materials were purchased as follows: restriction enzymes (Bethesda Research Laboratories and New England Biolabs), T4 DNA polymerase (P-L Biochemicals), DNase I (Sigma), radioisotopes (Amersham), rabbit anti-mouse IgM (Bionetics), rabbit anti-IgM (Tago), rabbit anti-λ (Miles), MOPC 104E an IgM ($\mu\lambda_1$) myeloma protein (Bionetics), calf intestinal alkaline phosphatase and S1 nuclease (Boehringer Mannheim). Unless otherwise stated cloning procedures were as described (10).

Oligodeoxyribonucleotides were synthesised by the phosphotriester procedures (11) and were designed to have the sequences; 5'-GATCAATGCAGGCTGTTGTG-3' (R45), and 5'-ATTCCTGAGTCACAACAGCC-3' (R44).

Bacterial strains and Plasmids

Plasmids were transformed into E. coli strain HB101, DH1, E. coli B (10) and E. coli K12 strain E103S (Dr. Lee Simon, Waksman Institute of Microbiology, Piscataway, New Jersey 08854-0759, personal communication), and grown in L-broth containing 0.1g carbenicillin per litre. Plasmids pABμ-11 (8) and pABλ1-15 (9) were a gift from Drs. A. Bothwell and D.

Baltimore. B1-8 proteins were gifts from Drs. M. Neuberger and T.Imanishi-Kari.

Pulse Chase Analysis

For pulse chase analysis inductions were set up as described above, except that the medium used consisted of: proline (0.3g/L), leucine (0.1g/L), Difco methionine assay medium (5g/L), glucose (60mg/L), thiamine (10mg/L), $CaCl_2$ (22mg/L), $MgSO_4$ (0.25g/L)m and carbenicillin (0.1g/L). During exponential growth cells were pulse labelled with 30 µCi/ml L-[^{35}S] methionine for 2 minutes, after which unlabelled methione (100 µg/ml) was added and the incubation continued for the times indicated.

Other Methods

Procedures used for bacterial lysis and fractionation were as described (12), as were procedures for inductions and protein assays (13).

Protein Purification

For further purification of the λ light chain, the cell debris were dissolved in 10mM Tris-HCl pH8.0, 25% formamide, 7M urea, 1mM EDTA and 2mM dithiothreitol. This material was loaded onto a DEAE Sephacel column (Pharmacia) (1 x 25cm at a flow rate of 5ml/hr) which had been equilibrated in 9M urea, 10mM Tris-HCl pH8.0, 1mM EDTA and 2mM DTT. The DEAE Sephacel column was developed using a 0-150mM NaCl gradient in loading buffer. The eluted peak of λ light chain immunoreactivity, corresponding to the major peak of protein was diluted to a final concentration of 2.25M urea, 10mM Tris-HCl pH8.0, 1mM EDTA, 2mM DTT and loaded onto an octyl-Sepharose column (Pharmacia) (2.5 x 10cm). Material was eluted by use of a urea gradient of 2.25-9M urea. The peak material was pooled, dialysed into ammonium bicarbonate and lyophilised.

The µ heavy chain was purified from 9M urea solubilised pellets by anion exchange chromatography and chromatofocussing (Pharmacia).

Reconstitution of Activity

Production of functional antibodies from E. coli expressing both heavy and light chains was achieved by lysing the cells and clarifying the supernatant by centrifugation. The insoluble material was washed, followed by sonication (3 times for 3 minutes), and finally dissolved in 9M urea, 50mM glycine-NaOH pH10.8, 1mM EDTA, and 20mM 2-mercaptoethanol. This extract was dialysed for 40 hours against 3 changes of 20 vols. of 100mM KCl, 50mM glycine-NaOH pH10.8, 5% glycerol, 0.05mM EDTA, 0.5mM reduced glutathione and 0.1mM oxidised glutathione. The dialysate was cleared by centrifugation at 30,000g for 15 minutes and loaded directly onto DEAE Sephacel, followed by

development with a 0-0.5M KCl linear gradient in 10mM Tris-HCl, 0.5mM EDTA, pH8.0.

The purified Ig μ and λ were treated as above, except that no anion exchange chromatography was carried out. The preparation was finally dialysed into phosphate buffered saline, 5% glycerol, 0.01% sodium azide and 0.5mM EDTA pH7.4.

RESULTS

Construction of Plasmids for Expression of λ Light Chain

We chose to express the λ gene in E. coli by direct expression of the gene lacking the eukaryotic signal peptide but containing a methionine initiator residue at the amino-terminus (met lambda). The approach used for bacterial synthesis of met-λ was to reconstruct the gene in vitro from restriction fragments of a cDNA clone and to utilise synthetic DNA fragments for insertion into the bacterial plasmid pCT54 (12) (figure 1). As a source of light chain we used the plasmid pABλ1-15 which contains a full-length λ_1 light chain cDNA cloned into the PstI site of pBR322 (9). We have previously outlined the construction of plasmids for the expression of λ light chain (14).

A plasmid was isolated (designated pCT54 19-1) and shown to have the anticipated sequence except that there was an alteration at the fifth codon from GTG to ATG, changing the amino acid at this point from valine to methionine (figure 1). Valine is an invariant residue at this position in mouse λ chains. Methionine, however, is the residue most frequently found in mouse κ chains at this position (15).

As most E. coli mRNAs have 6-11 nucleotides between the Shine-Dalgarno (SD) sequence and the AUG (16) the distance in pCT54 19-1 was reduced by modification at the ClaI site. Altering the distance between the SD sequence and the ATG has been demonstrated to alter the expression of a number of genes (13,14,17-20) presumably by placing the SD and ATG sequences in the optimal configuration for formation of the initiation complex. pCT54 19-1 was cut with ClaI and incubated with S1 nuclease. The amount of S1 nuclease was adjusted so that some DNA molecules would lose 1-2 extra base pairs as a result of 'nibbling' by the enzyme. This DNA on religation with T4 DNA ligase and transformation into E. coli strain HB101 gave rise to a number of plasmids which had lost the ClaI site. The nucleotide sequence across the modified region of two of these plasmids was determined (figure 1). pNP4 and pNP3 were shorter than pCT54 19-1 by 5 and 4 nuclectides

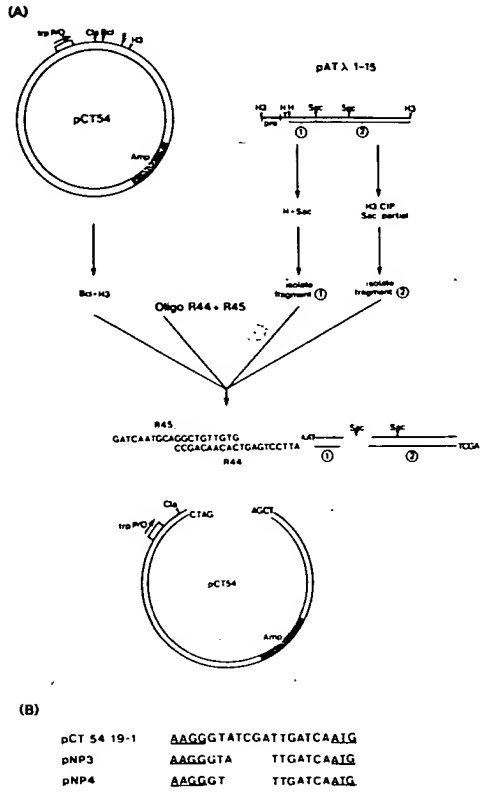

Figure 1. Construction of plasmids for the direct synthesis of λ light chain in E. coli.

Plasmid pATλ1-15 contains the λ gene inserted into the HindIII site of pAT153. (A) 5' HinfI - SacI fragment 1 was isolated by polyacrylamide gel electrophoresis. The 3' fragment 2 of the gene was isolated as a SacI - HindIII fragment. pCT54 was cut with BclI + HindIII and the λ gene fragments together with oligodeoxyribonucleotides R45 and R44 ligated to yield plasmid pCT54 19-1. (B) Digestion of pCT54 19-1 with ClaI and S1 nuclease produced plasmids pNP3 and pNP4, with reduced SD-ATG distances, E, EcoRI; H, HinfI; H3, HindIII.

respectively, giving SD-ATG distances of 9 and 10 nucleotides. Secondary structure analysis, as described (13), revealed no hairpin loop sequestering the SD or initiation codon into double-stranded regions of the mRNA of either pCT54 19-1 or the S1 derivatives. Such base pairing interactions have been shown drastically to reduce translational efficiency of a number of genes, notably those for phage λ cro (17), fibroblast and leukocyte

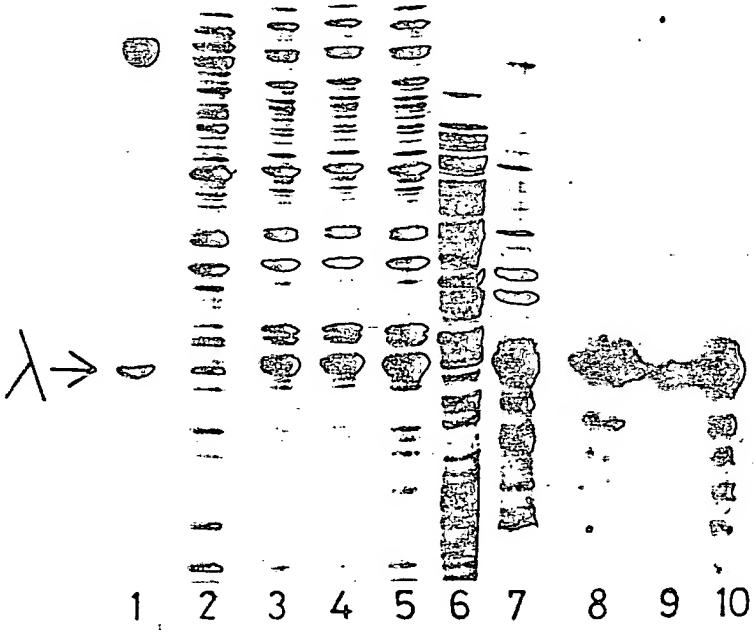

Figure 2. Accumulation and distribution of λ protein from E. coli E103S.
E103S cells containing pNP3 were grown under inducing conditions and samples taken throughout the induction cycle. Following electrophoresis, gels were stained with Coomassie blue (lanes 1-7) or subjected to analysis by Western blotting (lanes 8-10) using rabbit anti-λ serum and iodinated protein A (2μCi/ml). Lane 1, purified MOPC104E myeloma protein standard indicating the position of authentic λ protein; lane 2, E103S containing pNP3 after growth to stationary phase in L-broth; lanes 3-5, samples from E103S containing pNP3 taken at increasing absorbance during induction; lanes 6 and 7, respectively, soluble and insoluble fractions from pNP3 containing E103S; lane 8, unfractionated extract; lanes 9 and 10, respectively, soluble and insoluble fractions.

interferons (18), SV40 small-t antigen gene (19) and a murine μ heavy chain (13).

Expression of λ Protein

HB101 cells containing pNP3 were found to express λ light chain as determined by immunoprecipitation (14) and Western blot analyses. No such protein was evident in extracts from pCT54 19-1, cells containing pNP4 which had a SD-ATG distance one base pair shorter than that of pNP3 expressed only a very low level of λ light chain (14).

In the absence of specific immunoprecipitation a novel protein band was not visible from extracts of HB101 containing pNP3 nor was the λ protein

found to accumulate (data not shown). However, there was a dramatic difference when pNP3 was induced in the K12 strain E103S. In this strain λ protein was found to accumulate during induction until the cells reached stationary phase (figure 2, lanes 3-5) to a level of about 150 times that found in HB101 as determined by an ELISA (enzyme linked immunosorbent assay). These cells were found to contain inclusion bodies which appeared refractile under light microscopy, a phenomenon characteristic of high level expression of foreign proteins (21). An estimate of the percentage of total E. coli protein represented by recombinant λ protein was obtained by separating the proteins by gel electrophoresis, staining them with Coomassie blue and scanning the stained gel with a Joyce-Loebl chromoscan 3. This method showed that λ was the major protein present (figure 2, lane 5) and represented 13% of total E. coli protein. The λ protein had a half-life of 20 minutes in HB101 (data not shown) but accumulated to very high levels in E103S, suggesting that the lambda protein was much more stable in the latter strain. After cell lysis and centrifugation of HB101 or E103S containing pNP3, λ light chain was detected in the insoluble (figure 2, lanes 7 and 10) but not in the soluble fractions (figure 2, lanes 6), as determined by Coomassie blue staining. The identity of the major Coomassie blue stained band as λ protein was confirmed by Western blot analysis (figure 2, lanes 8-10). The presence of such immunoreactive bands was specific to pNP3 containing cells. When extracts from cells containing pCT70, a prochymosin expressing plasmid (12), were subjected to the same analysis, no bands were detected (data not shown). This more sensitive technique showed that a small amount of the λ protein was in the soluble fraction (figure 2, lane 9) The presence of a number of distinct immunoreactive proteins all smaller than full-length λ protein were also detected. These may result from proteolytic degradation of λ protein, from premature termination of transcription or from internal initiation of translation.

Expression of μ Protein

The construction of plasmids pNP11 and pNP14 for the expression of full-length mature μ protein under the control of the trp promoter has been described (13). E. coli B cells containing the μ expression plasmid pNP11 were grown under inducing conditions and soluble and insoluble extracts prepared, and analysed by SDS-PAGE. A novel band was seen after staining the gel with Coomassie blue in the lane containing proteins from the insoluble fraction (figure 3, lane 2). This band was not seen in the negative control lane which contained proteins from the same fraction from

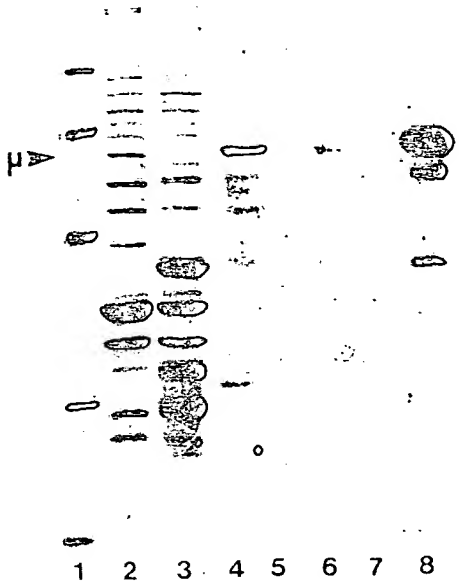

Figure 3. Expression and distribution of μ protein from E. coli B.
E. coli B cells containing pNP14 were grown under inducing conditions. Following electrophoresis, gels were stained with Coomassie blue (lanes 1-3) or subjected to analysis by Western blotting (lanes 4-8) using rabbit anti-IgM serum. Lane 1, molecular weight markers (94, 67, 43, 30 and 20Kd); lanes 2 and 4, respectively, pNP14 containing insoluble fraction; lanes 3 and 5, pCT70 containing insoluble fraction; lanes 6 and 7, respectively, pNP14 and pCT70 containing soluble fractions; lane 8, unfractionated sample from cells containing pNP14.

cells harbouring pCT70 (figure 3, lane 3). The novel band was found to migrate to a position corresponding to a protein of a molecular weight within less than 5% of the actual molecular weight of non-glycosylated μ of 62.5Kd. A duplicate set of lanes were transferred to nitrocellulose, and Western blotted. Alignment of the stained gel and the blot autoradiogram confirms that this novel band is antigenically related to IgM (figure 3, lanes 4 and 8). No band was found in extracts from cells containing pCT70 (figure 3, lanes 5 and 7). Only a low amount of μ was found in the soluble fraction (figure 3, lane 6).

A greatly increased level of expression of μ was found in E. coli B compared to HB101. Pulse chase analysis demonstrated that in E. coli B, a similar level of μ protein was detected after a 60 minute chase (figure 4, lane 3) as was seen after the initial labelling period (figure 4, lane 1). In HB101, however, very little μ protein could be seen after a 10

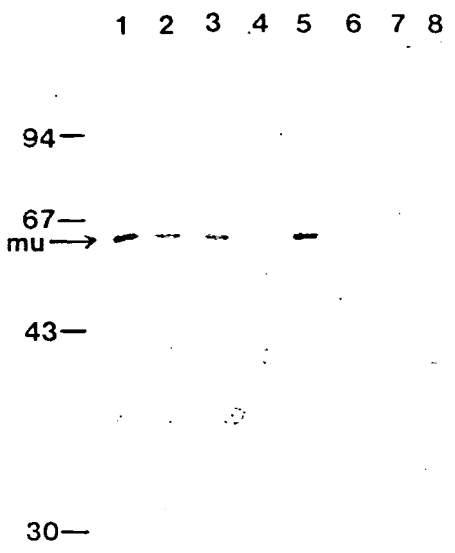

Figure 4. Pulse chase autoradiogram of pNP14 in E. coli B and HB101.
E. coli B and HB101 cells harbouring pNP14 were grown under inducing conditions and pulsed with L-[^{35}S]-methionine for 2 mins. after which unlabelled L-methionine was added to 100 µg/ml (zero time). Cells were harvested at varying times, and samples analysed by SDS-PAGE following immunoprecipitation. E. coli B: lane 1, zero time; lane 2, 30 min; lane 3, 60 min. Lane 4 is a zero time sample for pCT70 in HB101. HB101: lane 5, zero time; lane 6, 5 min; lane 7, 10 min; lane 8, 30 min.

minute chase (figure 4, lane 7), and none after 30 minutes (figure 4, lane 8), compared to the amount detected after the initial labelling period (figure 4, lane 5). Induced E. coli B cells harbouring pNP14 when examined by phase contrast microscopy were found to contain inclusion bodies.

Purification of Recombinant

The presence of λ light chain in the insoluble fraction was a useful purification step since it both concentrated the protein and separated it from the bulk of E. coli soluble proteins.

For further purification the E. coli insoluble material was solubilised in a Tris-HCl buffer containing 25% formamide and 7M urea and loaded onto a DEAE Sephacel column which had been preequilibrated in the same buffer with 9M urea, but without formamide. The bound material was eluted using a 0-150mM NaCl gradient in the urea buffer. The λ protein was the major eluted peak as determined by gel electrophoresis and ELISA. The λ protein was diluted to a final concentration of 2.25 urea and loaded onto an octyl-

Sepharose column. The λ protein bound and was eluted by using a 2.25-9M urea gradient. Following this step, only a single band of Coomassie blue stainable material corresponding to recombinant λ protein was visualised by SDS-PAGE (data not shown).

Expression of μ and λ Polypeptides in the same cell

Each of the Ig μ and λ genes in expression plasmids were transformed into the same E. coli cell to direct the synthesis of both Ig μ and λ polypeptides. In order to overcome plasmid incompatibility and provide a second antibiotic resistance marker, the trp promoter and λ sequences were excised from pNP3 on a HindIII-Bam HI fragment and inserted into the HindIII-Bam HI fragment of pACYC184 (10) to create pACYCλ. HB101 cells containing this plasmid were found to grow very poorly. This weak growth was thought to result from read through of RNA polymerase into the origin of replication, and inhibition of growth was virtually eliminated, by inserting the bacteriophage T7 early transcriptional terminator (22) at the HindIII site of pACYCλ. The resultant plasmid pACλ T7-1 has a chloramphenicol resistance gene and an origin compatible with the pBR322-derived origin on pNP14, the Ig μ expressing plasmid. Transformation of both plasmids into the same E. coli B cell was achieved in two steps, firstly pNP14 was introduced, and then pACλ T7-1, in two separate transformations to give ampicillin and chloramphenicol resistant clones.

E. coli B cells derived from double-transformant clones showed the presence of inclusion bodies and two novel polypeptide bands on stained gels of the insoluble fraction after lysis. These two bands correlated both with immunological activity by Western blotting for Ig μ and λ and their expected molecular weights of 63,500 and 25,000 daltons respectively (data not shown) Stability of the plasmids was also investigated and it was found that after 36 hours in shake flasks only 5% of the E. coli contained both antibiotic resistance markers, although 35% were carbenicillin resistance and 74% were chloramphenicol resistant. This illustrates the selective pressure against both plasmids together.

Reconstitution of Antigen Binding

It was of great interest to determine whether the concomitant expression of μ and λ would lead to the formation of functional IgM. In order to determine this extracts were made from E. coli containing both Ig μ and λ polypeptides and these tested for antigen binding. We used a two-site sandwich ELISA which detects μ chain binding to haptenalated bovine serum albumin (NIP-caproate-BSA). This assay demonstrates

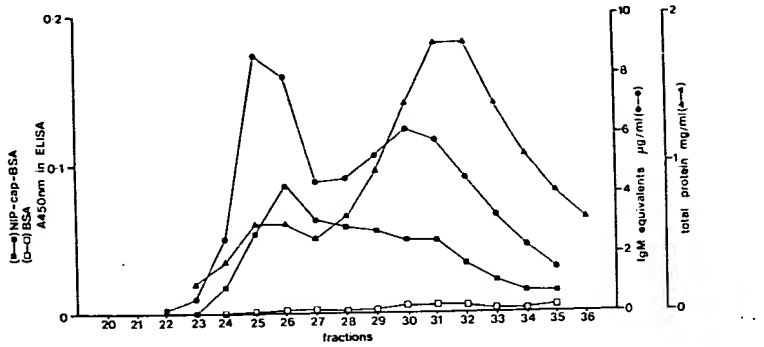

Figure 5. Purification of reconstituted antibodies.
Fractions from DEAE Sephacel anion exchange chromatography of E. coli B expressed Ig μ and λ. Analysis shows level of Ig μ expressed as B1-8 IgM equivalents; NIP-cap-BSA and BSA binding activities from ELISA's; total protein determined by A280nm readings (1.5 A280≡mg/ml).

sensitivity to 60 pg of B1-8 IgM. The extracts were prepared as soluble and insoluble material. The insoluble material was solubilized in the same buffer used in lysis but containing 8M urea followed by its dilution for assay. However, no antigen binding activity was detected.

In order to obtain activity for the Ig μ and λ, extracts were made of the insoluble fraction and these dialysed into buffer conditions in which disulphide interchange will occur at a higher frequency. The results from assays of material processed in this way indicated that some activity was obtained. The level of activity obtained in this way was too low to do any detailed studies on, so the resultant dialysate was purified by anion exchange chromatography (figure 5). This process resulted in the isolation of significant NIP-cap-BSA binding activity over that of background binding to BSA (figure 5). The assay of the fractions for the level of Ig μ, expressed as B1-8 IgM equivalents demonstrated two peaks of activity. This was not found to correlate with full length Ig μ by Western blotting (data not shown). The first peak observed may represent a fragment of Ig μ. The separation of NIP-cap-BSA binding activity from the majority of full length Ig μ and protein indicates that the hapten binding activity is contained within a particular molecular species formed at low efficiency.

The processing of insoluble material obtained from Ig μ expression in E. coli produced a similar IgM protein profile but without NIP-cap-BSA binding activity. This demonstrates that the activity recovered was a property of the combined immunoglobulin expression, not of some E. coli factor, or of the Ig μ heavy chain alone.

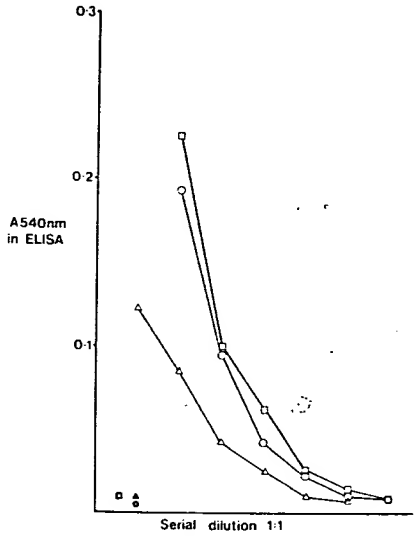

Figure 6. Specific hapten binding of reconstituted antibodies.
NIP-cap-BSA binding activity from fraction 26 (Δ), purified Ig μ and λ (□) and B1-8 (○). Binding in the presence of the 30μM NIP-cap. (▲,■,● respectively).

Further studies of the characteristics of the hapten (NIP-cap-BSA) binding were carried out. The reduction of hapten binding activity on dilution of renatured μ and λ expressed and purified together was less than that found for either B1-8 IgM or purified μ and λ expressed separately (figure 6). The dilution curves for B1-8 antibody and separately expressed μ and λ were virtually identical. Free hapten was found to inhibit most of the binding activity in both undiluted and diluted samples. Using B1-8 antibody as a standard for both IgM and hapten binding, the specific activity of the assembled antibody was calculated to be 1.4×10^4 gm/gm of IgM equivalents. This value demonstrates the inefficient recovery of activity, but possibly represents an underestimate of the specific activity due to an overestimate of full-length Ig μ in these fractions, as described above.

Heteroclitic Nature of Recombined Antibody

Detailed specificity of binding to NIP-cap-BSA was investigated by comparing the assembled antibodies with B1-8 IgM in the presence of free NIP-cap and NP-cap (figure 7). Both B1-8 IgM and the assembled antibodies showed that higher NP-cap than NIP-cap concentrations were required to inhibit NIP-cap-BSA binding.

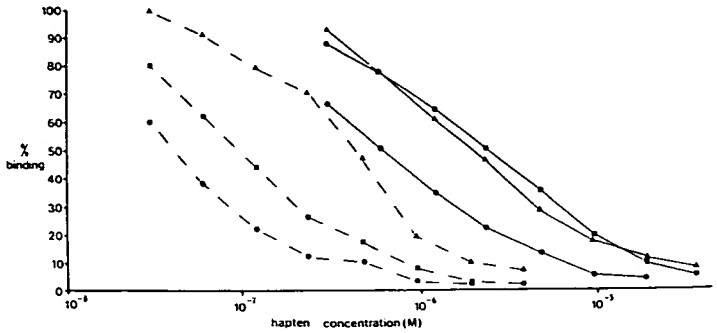

Figure 7. Heteroclitic binding of reconstituted antibody.
Binding of antibodies to NIP-cap-BSA; B1-8 IgM (■), fraction 26 (▲), purified Ig μ and λ (●), in the presence of free NIP-cap (---) or NP-cap (—).

The heteroclitic nature is demonstrated by the molar ratio of NIP to NP at 50% inhibition. The concentrations of NIP and NP at 50% inhibition (I50) were found to be similar for both B1-8 and the assembled antibodies (Table 1). Also the specificity ratios (NP I50/NIP I50) were similar (Table 1).

The specificity ratios for the μ and λ expressed together were somewhat lower than those found for B1-8 IgM or separately expressed μ and λ which were both very similar (Table 1). This is due to the identical concentrations of NP but the greater concentrations of NIP required to inhibit binding of μ and λ expressed together compared with B1-8 IgM. Although the specificity ratios for B1-8 IgM and separately expressed μ and λ are very similar, lower concentrations of NIP and NP are required for inhibition of separately expressed μ and λ compared to B1-8 IgM.

Table 1. Hapten concentration at 50% inhibition (I50) of binding of antibodies to NIP-cap-BSA solid phase.

	NIP I50 μM	NP I50 μM	NP I50 / NIP I50
B1-8 IgM	0.13 (SD, 0.05)	3.7 (SD, 2.9)	29
Fraction 26	0.34 (SD, 0.09)	1.9 (SD, 0.4)	6
Fraction 27 and 28	0.11 (SD, 0.02)	1.1 (SD, 0.3)	10
Purified μ and λ	0.04	0.84	22

SD = Standard Deviation.

DISCUSSION

Low level expression of λ and μ polypeptides was demonstrated in E. coli HB101. A greater level of expression of λ was found in strains E103S and E. coli B (J. Schoemaker, personal communication) and represented 13% total E. coli protein. A higher μ concentration was also found in E. coli B than HB101 cells containing pNP14 and was equivalent to 1% total E. coli protein. These differences in steady-state levels of the μ and λ proteins produced is most likely to result from different levels of protein stability. λ protein had a half life of 20 minutes in HB101 but was stable and accumulated in E103S. Similarly the pulse chase data shows that μ is more stable in E. coli B than HB101. This increased stability in E. coli B may be explained by the formation of inclusions, perhaps compartmentalizing μ away from proteases. E. coli B is known to be deficient in lon protease (23,24), so that the absence of this protease either acting upon μ, or being responsible for activating other proteases that act on μ, may be the major factor resulting in accumulation in E. coli B.

Both μ and λ proteins were expressed on compatible plasmids in the same cells. This expression of two different polypeptides necessary for the formation of a higher eukaryotic multi-subunit protein in E. coli represents the first of its kind. Thus it was of great interest to see if this concomitant expression of Ig μ and λ would lead to the formation of complete and functional IgM. No functional antibody was found following solubilisation of insoluble material. This indicated that the Ig μ and λ were not covalently interacting in the insoluble material in a specific way and that the inclusions represented non-active protein. The lack of activity correlated with insignificant amounts of Ig μ or λ in the soluble fraction. However, only a low percentage of cells were found to contain both plasmids after 36 hours induction. Thus the failure to find active (and presumably soluble) antibodies in vivo might simply be a reflection of the possibility that both polypeptides were not expressed at high levels in the same cells, and only a low percentage of cells contained both plasmids anyway.

Functional antibody activity, as defined by antigen binding, was obtained following dialysis of extracts in conditions in which disulphide interchange occurs. Significant antigen binding activity was recovered, which was abolished by competition with free hapten in a binding assay. Serial dilution of samples revealed a similar gradient in the reduction of binding compared to the monoclonal B1-8. The bacterially synthesised

antibody also demonstrated higher affinity binding to the related hapten NIP in a manner similar to that of the B1-8 antibody. Recovery of functional activity was not dependent on coexpression of the μ and λ polypeptide chains since results from purified μ and λ expressed from different cells showed similar results. Functional activity was, however, dependent on association between heavy and light chain polypeptides, since folding the μ heavy chain in the absence of λ light chain produced no antigen binding molecules.

Although the overall efficiency of production of functional antibodies was low, those molecules which did assemble, demonstrated activity in a way very similar to the hybridoma synthesised molecules. It is unclear why the efficiency of assembly was low, but was possibly due to the large number of disulphide bridges needed to assemble an IgM molecule. The heavy and light chain polypeptides were completely unfolded before assembly and so the intra-domain disulphide bridges as well as the inter-chain disulphide bridges need to be made correctly. Classically, when immunoglobulin heavy and light chains are separated and reassembled, only the inter-chain disulphide bridges are reduced and these molecules refold with high efficiency (25,26). The conditions used in our experiments for the formation of disulphide bridges were probably adequate, since the bacterially synthesised λ light chain when folded under such conditions led to the appearance of a discrete lower molecular weight species, as expected for accurately folded and oxidised λ light chain, which comigrated with oxidised λ protein from B1-8 (data not shown).

These results indicate that non-glycosylated immunoglobulin molecules produced in *E. coli* can be used for studies concerning antigen-binding. It is now possible to investigate the effects on antibody-antigen interactions following mutation of specific amino-acids of the antibody by site-directed mutagenesis and expression of the polypeptides in *E. coli*.

ACKNOWLEDGEMENTS

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